Purification and Characterization of the Endoglycosidase Heparanase 1 from Human Plantar Stratum Corneum: a Key Enzyme in Epidermal Physiology?

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A protein exhibiting endoglycosidase activity was purified from plantar stratum corneum to apparent homogeneity in two sequential column chromatographic steps. Protein sequencing revealed its identity with the recently cloned human heparanase 1, an enzyme, the expression of which is reported to be related to the metastasic potential of tumor cells. By using a heparanase 1 specific antibody we were able to demonstrate that, in the plantar stratum corneum, heparanase 1 exists in two forms, the active 50 kDa protein and the inactive 63 kDa form, probably a proform of the enzyme. The antibody also decorated numerous degradation fragments. Reverse transcription polymerase chain reaction studies as well as immunohistochemical analysis using reconstructed and normal human epidermis demonstrated clearly a keratinocyte differentiation related expression of heparanase 1. Interestingly, the antibody also strongly decorated dendritic cells, which after double labeling could be identified to be a subpopulation of the epidermal Langerhans cells. Based on our findings and the known history of this enzyme, we advanced the hypothesis that heparanase 1 has multiple physiologic functions in the epidermis: (i) it plays an important role in epidermal differentiation, possibly by modulating the liberation of heparan sulfate bound (growth) factors; (ii) in the stratum corneum, the endoglycosidase activity of heparanase 1 might be indispensable and represent the first step in the desquamation process; and (iii) in Langerhans cells, its catalytic activity is required for the trans-tissue migration of these cells. *Key words: heparanase/heparan sulfate/keratinocyte/Langerhans cells/stratum corneum. J Invest Dermatol 117:1266–1273, 2001*

Glycosaminoglycans (GAG) have been visualized by electron microscopy in the epidermis as rod-like granules attached to the keratinocyte cell surface. Heparan sulfate is the most abundant GAG, present throughout the epidermis (Tammi *et al*, 1987). Keratinocytes in culture are also able to synthesize a variety of GAG that attached to proteins form a large variety of proteoglycans (Rahemtulla *et al*, 1987; Piepkorn *et al*, 1990). Enzymatic deglycosylation of these proteins revealed that the majority of the core proteins are substituted with heparan sulfate (Haggerty *et al*, 1992). The exact function of epidermal GAG and proteoglycans is still unknown; however, they are suspected to be associated with a variety of functions, i.e., cell–cell interactions, cell adhesion, proliferation, differentiation, morphogenesis, remodeling of the extracellular matrix, hydration, antimicrobial activity (Ruoslahti, 1989; Ruoslahti and Yamaguchi, 1991), and modulation of the inflammatory response (Lider *et al*, 1990). In keratinocytes, heparan sulfate proteoglycans (HSPG) are supposed to modulate proliferation and differentiation by their ability to affect growth factor signaling and binding (Piepkorn *et al*, 1995; LaRochelle *et al*, 1999). HSPG might also play an important role in the process of desquamation as sugar moieties have been demonstrated to protect the corneodesmosomes from proteolysis. Walsh and Chapman (1991) described how a sequential action of glycosidases and proteases is required to degrade the corneodesmosomes.

In the hair follicle, the trans-membrane proteoglycan syndecan, which may possess both heparan and chondroitin sulfate chains, is suggested to be important for hair follicle morphogenesis (Couchman, 1993). The involvement of proteoglycans in hair growth has been deduced from the fact that in some diseases an increased presence of skin proteoglycans was associated with increased hair growth (Westgate *et al*, 1991).

Modulation of the physiologic functions of HSPG is attributed to the activity of endoglycosidases named heparanases. Heparanases are endo-β-D-glucuronidases that degrade GAG chains of HSPG at specific sites. Heparanase activities have been described in various human cell types and tissues, i.e., platelets, placenta, skin fibroblasts, smooth muscle cells, umbilical vein endothelial cells, macrophages, monocytes, and neutrophils (Freeman *et al*, 1999). Many studies were performed to investigate the role of human heparanase in association with the metastasic potential of tumor cells. Vlodavsky *et al* (1995) demonstrated that specific inhibitors of heparanase activity were able to block the invasive properties of heparanase expressing tumor cells and in consequence prevent metastasis.

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Abbreviations: GAG, glycosaminoglycan; Hpa1, human heparanase 1; HSPG, heparan sulfate proteoglycan; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; TGM, human transglutaminase.

Six research teams succeeded almost simultaneously to identify and clone the human heparanase (Hpa1) (Fairbanks *et al*, 1999; Hulett *et al*, 1999; Kussie *et al*, 1999; Toyoshima and Nakajima, 1999; Vlodavsky *et al*, 1999; Dempsey *et al*, 2000). Very recently Mckenzie *et al* (2000) reported the cloning of another human heparanase family composed of three splicing variants named Hpa2a, Hpa2b, and Hpa2c, with Hpa2a exhibiting a 35% homology with Hpa1.

Despite the presence HSPG in human skin, until now no epidermal heparanase has been identified. By using a modification of the previously described methods of Toyoshima *et al* (1999), we succeeded in identifying and purifying Hpa1 from human plantar stratum corneum. Here we describe its characterization and distribution in normal human epidermis and speculate on its possible role in epidermal physiology.

MATERIALS AND METHODS

Materials Vivaspin ultrafiltration filters were from Sartorius (Göttingen, Germany). Premade SDS-PAGE gelbond PAG film-supported gels for horizontal electrophoresis, Rainbow molecular weight markers, the silver staining kit, first-strand cDNA synthesis kit, HiTrap heparin sepharose columns, and gel filtration G75 HR16/60 columns were from Amersham Pharmacia Biotech (Uppsala, Sweden). Taq polymerase was from Sigma (St. Louis, MO, USA). The RNA preparation kit RNeasy was obtained from Qiagen (Hilden, Germany). Oligonucleotides were synthetized by Genset (Evry, France). Premade INSTA-blot human cell lines and human tissues were obtained from Calbiochem (San Diego, USA).

Heparanase assay We used a modification of the method previously described by Toyoshima *et al* (1999). Heparan sulfate, labeled with fluorescein isothiocyanate (FITC-HS), was used as a substrate and essentially prepared as described before; however, the gel filtration purification step was replaced by a washing procedure to remove unbound FITC and repetitive concentrations–dilutions on a 30 kDa cut-off Vivaspin ultrafilter (Sartorius) to eliminate low molecular weight FITC-HS (<30 kDa). After incubation at 37°C the samples were immediately separated, avoiding the use of heparin and the boiling of samples. The detection step by high performance liquid chromatography (HPLC) was replaced by a horizontal sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) performed on prefabricated gelbond PAG film-supported gels (12.5% acrylamide homogeneous gels, or 8–18% gradients) according to the manufacturer's instructions. Detection of FITC-HS and of its degradation products was performed on a Fluorimager laser scanner from Amersham Pharmacia Biotech.

Extracts from plantar stratum corneum and from reconstructed epidermis Reconstructed epidermis obtained after 13 d of culture and plantar stratum corneum from volunteers were homogenized in Tris-buffered saline (TBS), pH 7.2, containing 0.5% (wt/vol) Triton X100 and a protease inhibitor cocktail (complete EDTA-free from Roche Molecular used according to the manufacturer's instructions, complemented with 1 μM pepstatin) on an ice bed (500 μl buffer per 10 mg tissue weight) with a polytron for 1 min before centrifugation (10,000 × *g* for 10 min). Protein content of the supernatant was quantified using the Bio-Rad protein assay. Samples were adjusted to a protein concentration of 0.1 mg per ml before performing the heparanase assay.

Purification of heparanase Plantar stratum corneum of healthy donors (100 g) was homogenized with a polytron in 600 ml of TBS, pH 7.2, containing 0.5% (wt/vol) Triton X100 and protease inhibitor cocktail (complete EDTA-free from Roche Molecular used according to the manufacturer's instructions, complemented with 1 μM pepstatin) on an ice bed. The suspension was first filtered on a 40 μm mesh Nylon filter (Millipore). The resulting supernatants were successively passed over 1 μm and 0.2 μm Polycap filters before chromatographic separation.

All purification steps were performed at 4°C on a Biologic chromatography station (Bio-Rad). The extract (600 ml) obtained as described above was directly loaded on a Hitrap 5 ml heparin sepharose column previously equilibrated with sample buffer at a flow rate of 2.5 ml per min. The column was washed with five column volumes of sample buffer, where Triton X100 had been substituted by its low ultraviolet absorbing reduced form (buffer A). Elution was performed using the same buffer containing 1 M NaCl (buffer B) with a gradient from 0% to 100% of buffer B in 20 column volumes; fraction size was 1.05 ml. Protein elution was monitored by recording the OD_{280nm}. The eluted proteins were submitted to SDS-PAGE on prefabricated gelbond PAG film-supported gels (8–18% gradient gels) using a 1:2 dilution with 2 × Laemmli sample buffer and silver stained according to the manufacturer's instructions. The molecular weight standard used was a mix (1:1) of low and high molecular weight prestained Rainbow markers (Amersham Pharmacia Biotech) diluted 1:200 with 1 × Laemmli buffer. Fractions were assayed for heparanase activity using 10 μl of the fraction for 100 μl of the assay buffer (50 mM sodium acetate, pH 4.2) containing 2–4 μg of FITC-HS.

The heparin sepharose eluted fractions containing heparanase activity were pooled (fractions 64–73 included from heparin sepharose chromatography), and concentrated on a 10 kDa cut-off Vivaspin ultrafilter to a final volume of 2 ml. This sample was directly submitted to gel filtration separation on a G75 H16/60 column, previously equilibrated with the running buffer (50 mM sodium phosphate, pH 7, with 150 mM NaCl). Chromatography was performed at a flow rate of 1 ml per min with a fraction size of 0.5 ml. Fractions were analyzed by SDS-PAGE or used to determine heparanase activity.

Protein sequencing After acetone precipitation of the purified proteins (Méhul *et al*, 2000) the precipitated proteins were re-solubilized in 50 μl 1 × Laemmli buffer before electrophoresis. Horizontal SDS electrophoresis was performed on prefabricated gelbond PAG film-supported gels (12.5% homogeneous gels) according to the manufacturer's instructions. The molecular weight standard used was a mix (1:1) of low and high molecular weight prestained Rainbow markers (Amersham Pharmacia Biotech) diluted 1:4 with 1 × Laemmli buffer. The staining was performed with the premade Coomassie stain solution from Bio-Rad. The apparent molecular weights were calculated using Quantity One software from Bio-Rad. After staining, the gel was rinsed twice for 5 min in a MilliQ purified water bath and the protein bands were recovered with a scalpel. In-gel digestion by trypsin was performed as described by Kawasaki *et al* (1990). Peptides were separated by a tandem HPLC procedure (Kawasaki and Suzuki, 1990) combining an anion exchange HPLC on a DEAE Hemabio 1000 column (35 × 1.0 mm ID) from Interchrom (France) followed by a reverse phase HPLC on a C18 Uptisphere 3 μm ODSB column (150 × 1.0 mm ID) from Interchrom (France). Peptide sequencing was performed on an Applied Biosystem 494 device. Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry was performed on Applied Biosystems Voyager DEsuperSTR mass spectrometer.

Polyclonal antibody The antiheparanase polyclonal antibody was produced by the CovalAb Society (Lyon, France) by immunizing rabbits with selected peptide from the Hpa1 sequence. One peptide was retained as potentially immunizing: DB16CA (KLYGPDVGQPRRKTA, amino acid residues 264–278 from Swissprot sequence Q9UL39). The rabbits were immunized with a proprietary protocol (CovalAb) and the antibody was finally affinity purified. Antibody concentration obtained after the immuno-purification step was 83 μg per ml.

Reconstructed human epidermis Reconstruction of human epidermis with normal human keratinocytes was performed as described previously (Tinois *et al*, 1991). The reconstructed epidermis was examined at different times during its reconstruction, starting 1 d after seeding the cells to day 15 of its culture. To evaluate the effect of retinoic acid the medium was supplemented with 1 μM retinoic acid between days 4 and 11 with a medium change each second day. Reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis were performed at the end of the treatment. Untreated cultures served as control.

Western blot analysis Epidermis from plastic surgery and plantar stratum corneum were homogenized in 1 × Laemmli buffer containing 2% (wt/vol) SDS and 200 mM dithiothreitol (500 μl buffer per 10 mg tissue weight) with a polytron for 1 min and boiled 10 min before centrifugation (10,000 × *g* for 10 min). Proteins in the supernatant were quantified using the Bio-Rad protein assay to adjust samples to 0.5 mg protein per ml. Samples from purification steps were diluted 1:1 with 2 × Laemmli buffer and boiled for 10 min before use. Fifteen microliter samples were run on 12.5% SDS-PAGE gels. Proteins were transferred to a PVDF membrane (Immobilon P, Millipore) in the presence of 20 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% SDS, and 20% (vol/vol) methanol using a *trans*-blot cell (Bio-Rad) with a constant voltage of 60 V for 4 h. The ready to use blot containing extracts from different

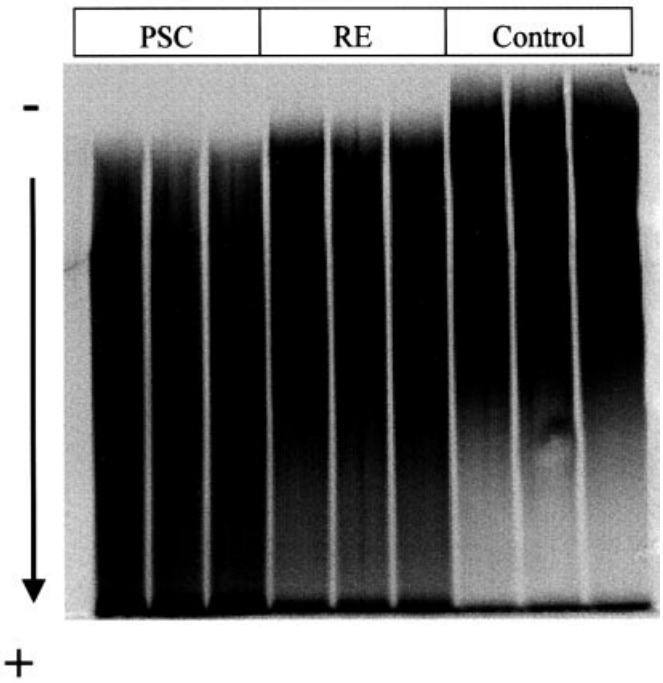

Figure 1. Heparanase activity in extracts from plantar stratum corneum and reconstructed epidermis. The activity was detected using an FITC-HS based assay (see *Materials and Methods*). SDS-PAGE of FITC-HS was run on 12.5% acrylamide gel after a 24 h incubation at 37°C in the presence of native extracts from plantar stratum corneum and from reconstructed epidermis. Control represents heparan sulfate without any extract. Experiments were performed in triplicate with the same extract. Fluorescence was detected on a Fluorimager scanner. The shift toward lower molecular weight of FITC-HS compared to control signals the presence of heparanase activity.

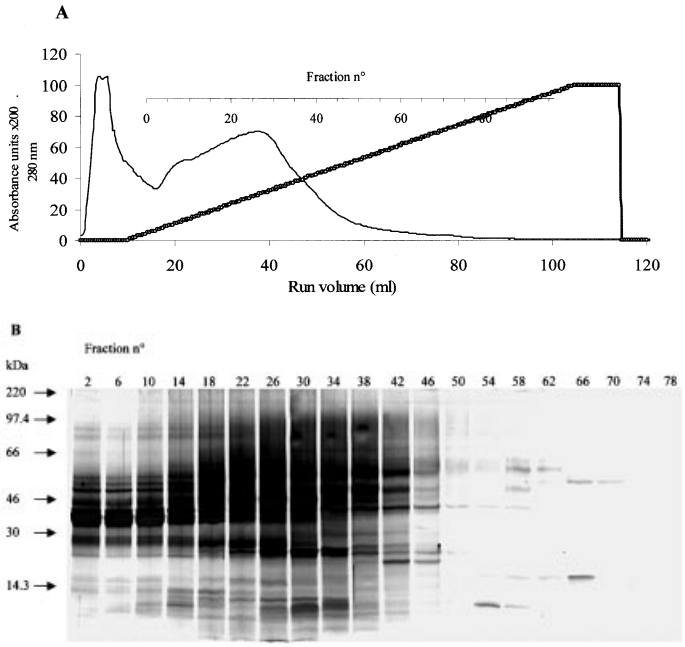

Figure 2. Heparin sepharose chromatography of plantar stratum corneum extract. (*A*) Heparin sepharose chromatography. Solubilized proteins (150 mg) were loaded on the column and eluted with an NaCl gradient 0 to 1 M (○). Protein elution was monitored at 280 nm (3/4). (*B*) SDS-PAGE analysis. Fractions (7.5 μl) were separated by SDS-PAGE electrophoresis (12.5% acrylamide gel) and silver stained. *Arrows* indicate the molecular weight markers.

human tissues (Insta-blot, Calbiochem) was prewetted with methanol and rinsed twice with water before applying the detection procedure. The filters were blocked with 25 mM Tris-HCl, pH 7.2, 150 mM NaCl, and 0.05% (vol/vol) Tween 20 containing 0.5% nonfat milk (TBSTL), at room temperature for 1 h. Thereafter, filters were incubated overnight at 4°C with the primary monoclonal antibody DB16CA diluted 1:500 in TBSTL. The filters were then washed twice for 5 min in TBSTL and incubated at room temperature for 2 h with the second antibody coupled to peroxidase (Sigma), diluted in TBSTL as indicated by the supplier. The filters were washed twice for 5 min in TBS before the detection procedure. The bands were visualized using the ECL+ substrate system (Amersham Pharmacia Biotech) on a FluorSmax imager (Bio-Rad). Pre-stained Rainbow molecular weight markers served as standards and molecular weights were calculated by using the Quantity-One software (Bio-Rad). For the Insta-blot, the Amido black colored molecular weight markers on the filter were used as reference.

RT-PCR analysis Total RNA was obtained from reconstructed human epidermis during its differentiation between days 1 and 15. RNA was prepared using an RNeasy kit. Briefly, two samples of the reconstructed epidermis (1 cm^2 each) were homogenized in 300 μl of lysis buffer (included in the kit) and the cell debris was removed using a QIA shredder column. cDNA was produced from purified total RNA using the First-Strand cDNA Synthesis Kit. Expression analysis of Hpa1, human calmodulin, loricrin, transglutaminase 1 (TGM1), transglutaminase 3 (TGM3), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed by RT-PCR using equivalent amounts of total RNA (0.1 μg per assay), Taq polymerase, and different primers (for Hpa1, 5′-caagaaggaatcaacctttgaag-3′ and 5′-gtagtccaggagcaactgagc-3′; for calmodulin, 5′-ataaagatggcgatggcaccat-3′ and 5′-ccattgccatccttgtcaaaga-3′; for loricrin, 5′-accacggaggcgaaggagttt-3′ and 5′-ctggggttgggaggtagttg-3′; for TGM1, 5′-gcggcaggagtatgttctta-3′ and 5′-agggatgtgtctgtgtcgtg-3′; for TGM3, 5′-ctgcgtgctgatggtggag-3′ and 5′-tcattcggctacgtcgatg-3′; for GAPDH, 5′-aatcccatcaccatcttcca-3′ and 5′-gtcatcatatttggcaggtt-3′) under the following conditions: one cycle for 2 min at 95°C, 25–30 cycles at 94°C for 30 s, 55°C–60°C for 30 s, 72°C for 30 s, and one cycle at 72°C for 2 min in parallel. The resulting PCR products were visualized by Gelstar staining after separation on 2%–3% (wt/vol) agarose gels.

Histochemistry Thin cryosections (5 μm) of skin from plastic surgery were obtained with a Microm HM500M microtome. Sections were fixed in acetone at –20°C for 5 min and air dried.

Immunohistochemical detection of Hpa1 was performed with streptABComplex/HRP kit from Dako and AEC detection kit from Sigma according to the manufacturer's instructions except that the incubation steps were prolonged to 1 h using DB16CA diluted 1:2 in phosphate-buffered saline/Tween 20 0.05% (PBST) as primary antibody and an antirabbit biotinylated secondary antibody from Roche Molecular diluted 1:200 in PBST. Nuclei were counterstained with hematoxylin. Immunohistochemical detection of heparan sulfate was performed by direct labeling with an antiheparan sulfate fluorescein conjugate antibody from Calbiochem (cat. no. 375081) diluted 1:2 in PBS.

The double staining immunofluorescence detection of CD1a positive Langerhans cells and Hpa1 positive cells was performed as follows. Sections were washed in PBS for 5 min before and after acetone fixation; thereafter nonspecific binding sites were blocked for 20 min in PBS containing 2% bovine serum albumin and then for 20 min in PBS containing 0.2% bovine serum albumin. Sections were then incubated for 1 h in a mixture containing a 1:50 dilution of a mouse monoclonal antibody to human CD1a (Becton Dickinson no. 347430) and a 1:2 dilution of the rabbit immunopurified polyclonal antibody DB16CA specific for Hpa1. After incubation and two washes in PBS containing 0.2% bovine serum albumin, sections were incubated in a mixture containing a 1:64 dilution of an antimouse FITC conjugate antibody (Sigma no. F-2012) and a 1:400 dilution of an antirabbit-cy3 conjugate antibody (Sigma no. C-2306). After two washing steps for 5 min in PBS containing 0.2% bovine serum albumin the sections were mounted in a mounting medium from Dako (no. S3023) and examined using a DMR microscope (Leica).

RESULTS

Heparanase purification Heparanase activity in extracts obtained from plantar stratum corneum and reconstructed human

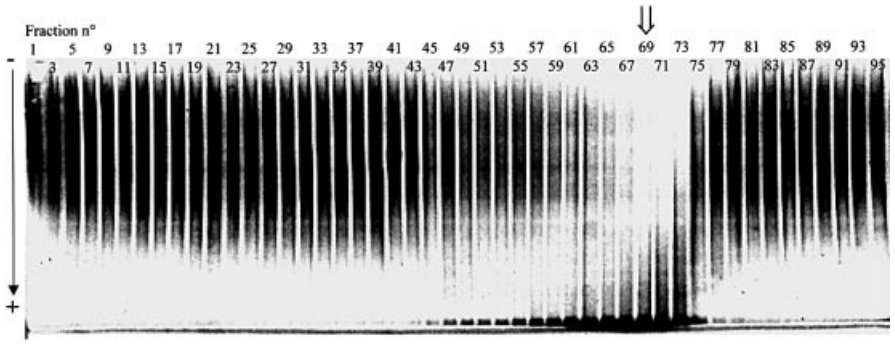

Figure 3. Gel-shift heparanase activity assay of the fractions obtained after affinity chromatography. Heparanase activity was assayed using the FITC-HS based assay for each fraction (see *Materials and Methods*). SDS-PAGE of FITC-HS was performed on an 8–18% acrylamide gel after 1 h incubation at 37°C with the heparin sepharose fractions. Fluorescence was detected on a Fluorimager scanner.

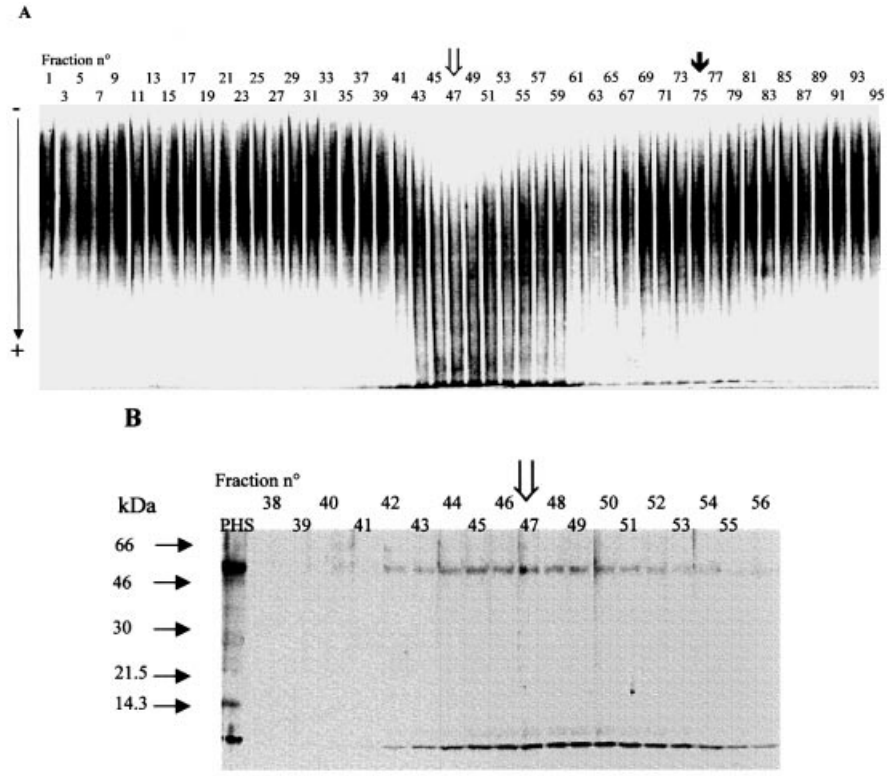

Figure 4. Analysis of the fractions obtained after sepharose G75 gel filtration. (*A*) Heparanase activity detection after separation of the pooled fractions on a G75 HR16/60 column. Heparanase activity was detected using the FITC-HS based assay. SDS-PAGE of FITC-HS was run on an 8–18% acrylamide gel after 30 min incubation at 37°C. Fluorescence was detected on a Fluorimager scanner. The fraction with maximum activity is marked with an *open arrow*. A second activity peak can also be detected and is marked with a *black arrow*. (*B*) SDS-PAGE analysis. Fractions with the maximum heparanase activity were separated on an 8–18% acrylamide gel. The gel was silver stained. *PHS lane* represents the pooled heparanase active fractions after heparin sepharose affinity chromatography of the crude extract (see **Fig 3**).

epidermis could clearly be detected using the FITC-HS based heparanase assay (**Fig 1**). Separation of the plantar stratum corneum extract by affinity chromatography on a heparin sepharose column (**Fig 2*A***) revealed that the majority of the proteins exhibit low affinity for heparin sepharose and could be eluted with NaCl concentrations below 0.5 M. We expected heparanase activity to be eluted with NaCl concentrations above 0.5 M and to be present in the fractions containing less proteins, i.e., fractions higher than number 50 (see **Fig 2*B***: SDS-PAGE separation of the affinity column fractions). Indeed, heparanase activity was detected in fractions 63–73 with a clear peak in fraction 69 (**Fig 3**). For further purification, the heparanase-containing fractions were pooled to perform gel filtration on a G75 sepharose column. The resulting fractions were analyzed for their heparanase activity (**Fig 4*A***). Those exhibiting activity were further analyzed by SDS-PAGE (**Fig 4*B***). Following G75 sepharose column separation and using the calibration curve obtained with standard proteins, we calculated molecular weights of 45 kDa and 21 kDa for the major heparanase active fractions (**Fig 4*A***, fractions 47 and 75, respectively). After the subsequent SDS-PAGE separation of fractions 38–56 (**Fig 4*B***), two proteins were detected, one with a molecular weight of 50 kDa and one with 8 kDa, probably representing the two subunits forming the heterodimeric structure of activated Hpa1 as described by Fairbanks *et al* (1999). The above-mentioned 21 kDa protein exhibiting heparanase activity could not be detected by SDS-PAGE (result not shown).

Heparanase identification Sequence analysis after concentration of the proteins in the highly active fractions by acetone precipitation confirmed the identity of the 50 kDa protein with Hpa1. Three peptides, separated by HPLC after trypsinolysis of the concentrated proteins and sequenced following the Edman protocol, revealed the following sequences: (i) AGGEVIDSVT (amino acid residues 287–296 from Swissprot sequence Q9UL39 for Hpa1); (ii) KADIFINGSQLGEDFIQLHK (amino acid residues 234–253); and (iii) PLRPGSSLGLPAFSYSFFVIRNAK (amino acid residues 517–540) (**Fig 5**). For the 8 kDa band, a MALDI-TOF mass spectrometry analysis of the peptides generated by trypsinolysis was performed. Mass fingerprinting research in the databases led to the identification of three peptides belonging to the small subunit of Hpa1 and representing 23% of the sequence of the little subunit described by Fairbanks *et al* (1999) (**Fig 5**).

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        10         20         30         40         50
         |          |          |          |          |
MLLRSKPALPPPLLMLLLLGPLGPLSPGALPRPAQAQQDVVDLDFFTQEP  50
        60         70         80         90        100
         |          |          |          |          |
LHLVSPSFLSVTIDANLATDPRFLILLGSPKLRTLARGLSPAYLRFGGTK 100
----------------------SSSSSSSSS------SSSSSSSS-----
----------------------PPPPPPPPP------PPPPPPPP-----
       110        120        130        140        150
         |          |          |          |          |
TDFLIFDPKKESTFEERSYWQSQVNQDICKYGSIPPDVEEKLRLEWPYQE 150
SSSSSSSSS-----------------------------------------
PPPPPPPPPPPPPPPPP-------------PPPPPPPPPPP--PPPPPPP
       160        170        180        190        200
         |          |          |          |          |
QLLLREHYQKKFKNSTYSRSSVDVLYTFANCSGLDLIFGLNALLRTADLQ 200
PPPPPPPPPPPPP-------------------------------------
       210        220        230        240        250
         |          |          |          |          |
WNSSNAQLLLDYCSSKGYNISWELGNEPNSFLKKADIFINGSQLGEDFIQ 250
---------------------------------EEEEEEEEEEEEEEEEE
       260        270        280        290        300
         |          |          |          |          |
LHKLLRKSTFKNAKLYGPDVGQPRRKTAKMLKSFLKAGGEVIDSVTWHHY 300
EEE-------------------------------EEEEEEEEEE-----
------PPPPPPPPPPPPPPPPPP-------------PPPPPPPPPPPPP
--------------LLLLLLLLLL-------------LLLLLLLLLLLLL
       310        320        330        340        350
         |          |          |          |          |
YLNGRTATREDFLNPDVLDIFISSVQKVFQVVESTRPGKKVWLGETSSAY 350
PPPPP----PPPPPPPPPPPPPPPPPPPPPPPPPPPPPP-----------
LLLLL----------------------LLLLLLLLLLLLL-----------
       360        370        380        390        400
         |          |          |          |          |
GGGAPLLSDTFAAGFMWLDKLGLSARMGIEVVMRQVFFGAGNYHLVDENF 400
--------------------PPPPPPPPPPPPPP-----------------
       410        420        430        440        450
         |          |          |          |          |
DPLPDYWLSLLFKKLVGTKVLMASVQGSKRRKLRVYLHCTNTDNPRYKEG 450
       460        470        480        490        500
         |          |          |          |          |
DLTLYAINLHNVTKYLRLPYPFSNKQVDKYLLRPLGPHGLLSKSVQLNGL 500
-----------------PPPPPPPP----PPPPPPPPPPPPPP-------
       510        520        530        540        550
         |          |          |          |          |
TLKMVDDQTLPPLMEKPLRPGSSLGLPAFSYSFFVIRNAKVAACI      545
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Figure 5. Alignment of sequence data obtained by Edman sequencing and MALDI-TOF mass spectrometry from the purified heparanase with the published amino acid sequence of Hpa1 (Swissprot sequence Q9UL39). Italic letters indicate the signal peptide position. Bold letters indicate the protein part that is eliminated as the activation occurs leading to the heterodimeric active form of the enzyme, i.e., the association between the 8 kDa fragment (*double underlined*) and the 50 kDa fragment (*single underlined*) (Fairbanks *et al*, 1999). «E» indicates the amino acid sequences obtained by Edman sequencing of the peptides generated by trypsin from the 50 kDa purified protein. Peptides generated by trypsin from purified proteins and with masses obtained by MALDI-TOF mass spectrometry that are exactly matching the theoretical masses calculated from the published sequence are indicated as follows: «p» peptides from the 63 kDa band, «L» peptides from the 50 kDa band, and «S» peptides from the 8 kDa band. The peptide used for the DB16CA polyclonal antibody development is boxed.

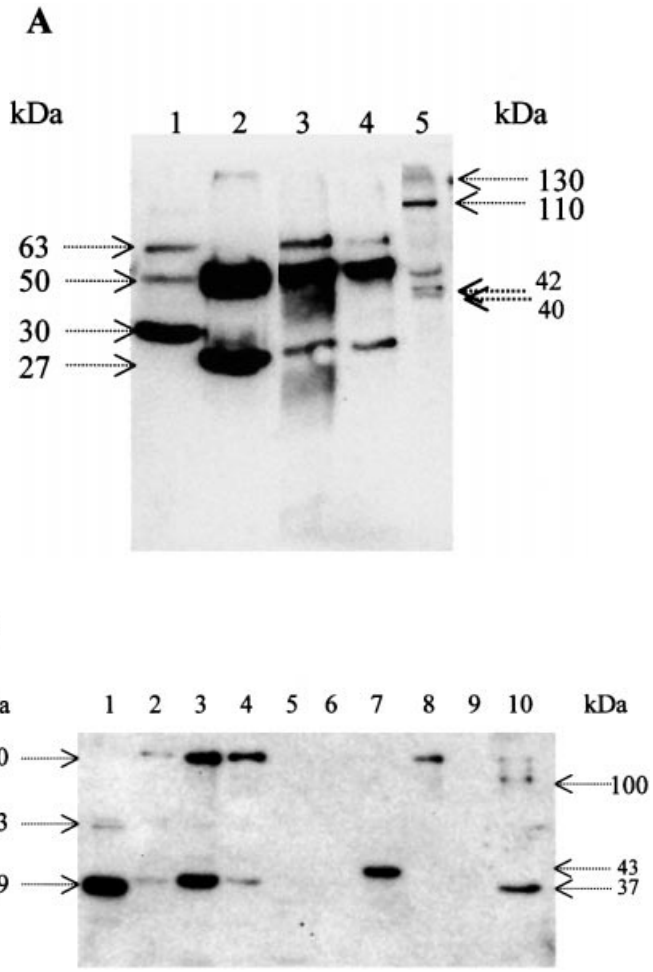

Figure 6. Western blot analysis using the polyclonal antiheparanase antibody DB16CA. (*A*) *Lane 1*, plantar stratum corneum; *lane 2*, fraction number 69 after heparin sepharose chromatography (see **Fig 3**); *lane 3*, pooled fractions (63–73) after heparin sepharose chromatography; *lane 4*, fraction number 47 after gel filtration chromatography; *lane 5*, total extract from human epidermis. (*B*) Human tissues INSTA-blot: *lane 1*, brain; *lane 2*, heart; *lane 3*, small intestine; *lane 4*, kidney; *lane 5*, liver; *lane 6*, lung; *lane 7*, skeletal muscle; *lane 8*, testis; *lane 9*, spleen; *lane 10*, pancreas. Calculated molecular weights are indicated on the left and on the right.

Characterization of Hpa1 using Western blot analysis

(a) Hpa1 from the plantar stratum corneum extracts and purified fractions In the plantar stratum corneum extracts, Western blot analysis with the DB16CA antibody revealed three bands (**Fig 6*A***, *lane 1*): a 63 kDa band representing probably the inactive proform of the enzyme; a 50 kDa band, the active form as previously described by Fairbanks *et al* (1999); and a 30 kDa band, representing probably a degradation product. MALDI-TOF mass spectrometry analysis of the peptides generated from the 63 kDa band provided a good probability for its identity with the proform of Hpa1 (11 peptides representing 29% of the protein; see **Fig 5**). Analysis of fraction 69, containing the highest heparanase activity after heparin sepharose chromatography separation (see **Fig 3**), revealed the 50 kDa enzyme and a new 27 kDa form (**Fig 6*A***, *lane 2*). Western blot analysis of the pooled fractions (fractions 63–73) made the 63 kDa proform of heparanase reappear (**Fig 6*A***, *lane 4*).

As expected, the gel filtration chromatography step following heparin sepharose chromatography contributed to a further purification without affecting the immunodetection pattern, indicating that under native conditions the 63, 50, and 27 kDa proteins can form complexes (**Fig 6*A***, *lane 4* compared with *lane 3*).

(b) Hpa1 from human tissue extracts The Western blot analysis of an extract prepared from normal human epidermis showed the presence of multiple bands including the 50 kDa form of Hpa1 (**Fig 6*A***, *lane 5*). The identity and exact nature of the low molecular bands (40 and 42 kDa) as well as that of the high molecular weight 110 and 130 kDa are under investigation. It was interesting to observe that some high molecular weight bands detected in the Western blot of human epidermis are also present in

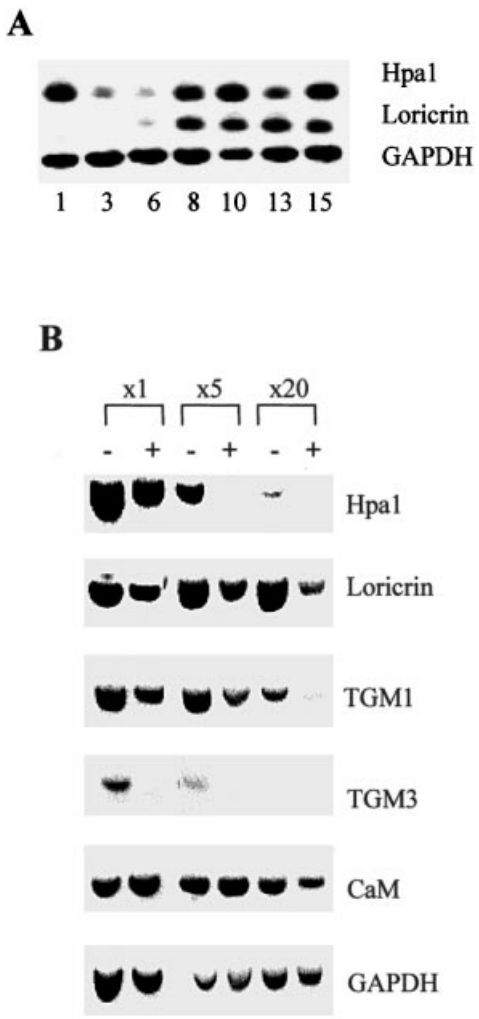

Figure 7. RT-PCR analysis of Hpa1 expression. (*A*) Hpa1 expression in keratinocytes during the reconstruction of human epidermis. (*B*) Effect of a 1 μM retinoic acid on Hpa1 expression in reconstructed human epidermis. The figure shows the band intensity of specific amplicon generated by a set of primers corresponding to differentiation markers (loricrin, TGM1, TGM3) and of two housekeeping genes (calmodulin, GAPDH) compared with Hpa1 (see *Materials and Methods*). The total mRNA (0.1 μg undiluted) was diluted 5× or 20× to evaluate more accurately the respective mRNA levels.

other human tissues (**Fig 6*A***, *lane 5*, compared with **Fig 6*B***, *lanes 2, 3, 4, 8, 10*); however, none of the INSTA-Blot™ tissues demonstrated detectable levels of the 50 kDa heparanase, which seems to be abundantly expressed in plantar stratum corneum, a tissue composed of corneocytes, the terminal state of keratinocyte differentiation.

Hpa1 expression is related to keratinocyte differentiation To investigate a possible differentiation-related expression of Hpa1 in the epidermis, we performed RT-PCR analysis in keratinocytes at different time intervals during the reconstruction of a human epidermis. The time course of Hpa1 expression (**Fig 7*A***) revealed a strong expression at day 1, followed by a remarkable reduction to almost absence at day 6. Hpa1 expression was very high again at day 8, matching the expression pattern of loricrin, a classical marker of keratinocyte differentiation.

Retinoic acid is known to stimulate keratinocyte proliferation and to downregulate markers of epidermal differentiation (Schmidt *et al*, 1989). A comparative RT-PCR analysis of reconstructed epidermis exposed to 1 μM retinoic acid during days 4 and 11 of its culture *versus* an nontreated sample clearly revealed that Hpa1 expression is strongly reduced in the presence of retinoic acid (**Fig 7*B***). This downregulation of Hpa1 expression was also confirmed by Western blot analysis to occur at the protein level (data not shown).

Immunolocalization of Hpa1 in normal human epidermis **Figure 8**(***A***) reveals Hpa1 expression in the upper part of the epidermis, ranging from the stratum granulosum into the first layers of the stratum corneum. In the deeper epidermis, the DB16CA antibody strongly decorated a dendritic cell population, which, due to their suprabasal localization, were suspected to be Langerhans cells. This asumption could be confirmed by double staining immunofluorescence experiments (**Fig 9**). The exact nature of this Hpa1 positive Langerhans cell subpopulation is under investigation. Immunolocalization of heparan sulfate (**Fig 8*B***) confirmed the presence of the heparanase substrate at the basement membrane between dermis and epidermis and in the dermis but also showed a faint staining in the suprabasal layers of the epidermis.

DISCUSSION

The endoglycosidase heparanase is an enzyme that cleaves heparan sulfate, a major component of the extracellular matrix and basement membranes. Its expression in mammalian cells is generally associated with the mediation of tumor metastasis and angiogenesis (Vlodavsky *et al*, 2000) and its mRNA expression level is used as a diagnostic parameter to evaluate the metastatic potential of tumor cells (Ikuta *et al*, 2001). So far no heparanase activity has been described in epidermal cells.

At the base of our investigations was a research program designed to elucidate the sequence of events implicated in the control of epidermal desquamation, a process mainly controlled by proteases that degrade the corneodesmosomal proteins and in consequence liberate the corneocytes. Sugars have also been described to modulate desquamation, however. Walsh and Chapman (1991) demonstrated clearly in their model that the proteolytic degradation of corneodesmosomes is preceded by endoglycosidase activity, a process that they defined as the first step in desquamation. Numerous exoglycosidases have been reported in epidermis (Nemanic *et al*, 1983) but their implication in the desquamation process has remained questionable.

By sequencing human stratum corneum proteins exhibiting heparanase activity after a two-column step purification process, we were able to demonstrate the presence of what might be the missing link in the desquamation cascade, the endoglycosidase heparanase (Hpa1). Using specific antibodies generated for the study, we could confirm the presence of Hpa1 in the stratum corneum of human skin, an acidic environment (Ohman and Vahlquist, 1994) particularly favorable for Hpa1, which exhibits a pH optimum between 4 and 5. Western blot analysis revealed the presence of two Hpa1 proteins in the plantar stratum corneum, the 50 kDa active form of the enzyme and its proform with 63 kDa accompanied by lower molecular weight forms that we suppose to be degradation products. A more detailed analysis of purified Hpa1 (**Fig 4*B***) demonstrated that, as already described by Fairbanks *et al* (1999) for human platelet heparanase, epidermal active Hpa1 has a heterodimeric structure and is composed of an 8 and a 50 kDa chain.

Evidence that Hpa1 expression in human epidermis is not related to any transformation process but closely related to keratinocyte differentiation was obtained by RT-PCR studies during the reconstruction of human epidermis and immunohistochemical analysis of normal human epidermis. After day 6 of the reconstruction, Hpa1 expression matched the expression of loricrin, a classical marker of late keratinocyte differentiation. The relatively high Hpa1 expression in keratinocytes at day 1 probably indicates a role of this enzyme in the migration of freshly seeded keratinocytes to cover the surface of the dermal substrate. One might speculate

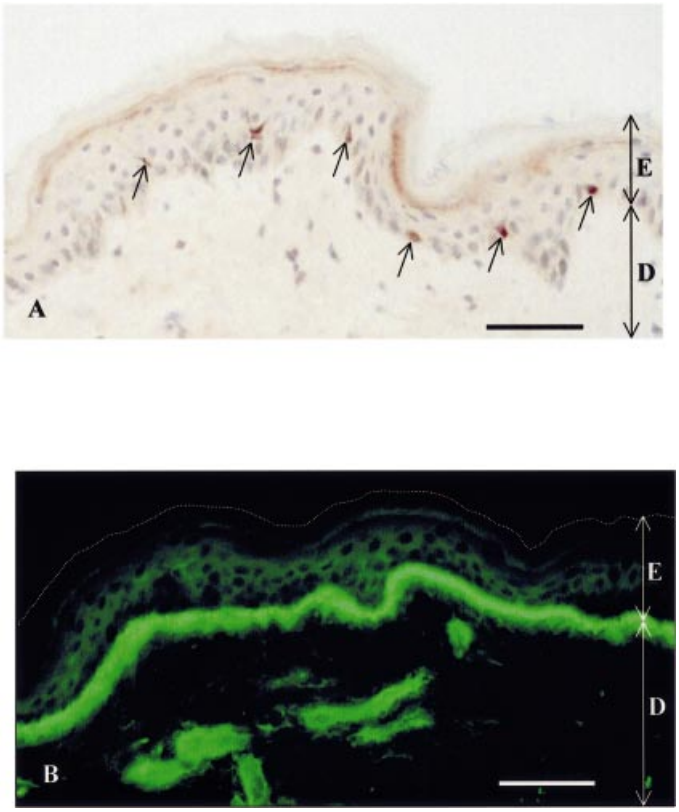

Figure 8. Comparison of the immunolocalization of heparanase and heparan sulfate in epidermis. (*A*) Immunohistochemistry using rabbit polyclonal antibody DB16CA against heparanase as the primary antibody. *Arrows* indicate intensively decorated cells in the suprabasal layers of the epidermis, probably Langerhans cells. (*B*) Immunofluorescence using FITC-labeled mouse monoclonal antibody against heparan sulfate. The *dotted line* indicates the stratum corneum limits. E, epidermis; D, dermis. *Scale bar*: 100 μm.

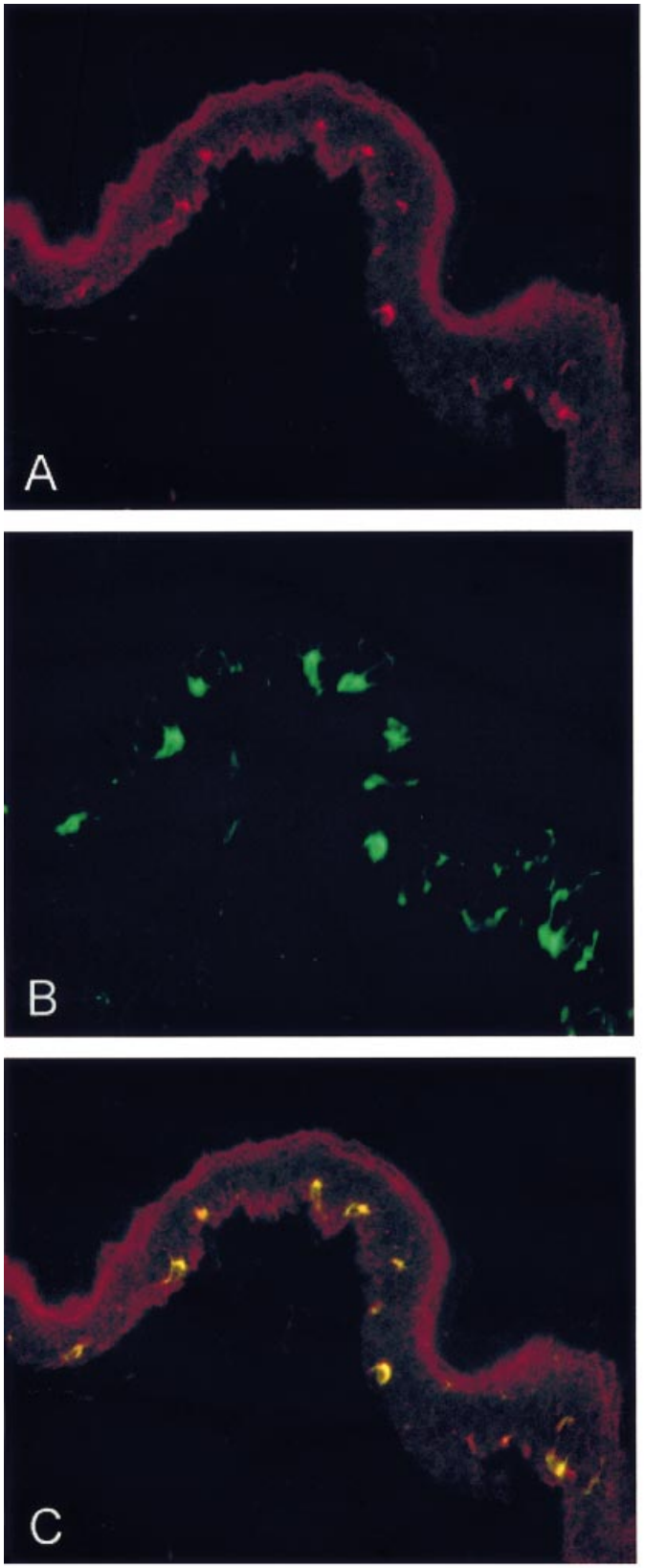

Figure 9. Double–staining immunofluorescence microscopy of epidermis using antiheparanase and anti-CD1a antibodies. Distribution of Hpa1 (*A*, red immunofluorescence) in the upper part of the epidermis and in dendritic cells was confirmed. A great number of CD1a positive Langerhans cells (*B*, green immunofluorescence) were detected in the suprabasal layers of the epidermis. The yellow mix color (*C*) in the dendritic cells evidenced that a part of the Hpa1 detected in the epidermis is produced by a subpopulation of Langerhans cells.

that Hpa1 might also play an important role in keratinocyte migration during wound healing.

It was interesting to observe that, besides the stratum corneum, the Hpa1 specific antibody decorated a subpopulation of epidermal Langerhans cells. After exposure to haptens, these dendritic cells migrate to the peripheral lymph nodes to present the captured and processed antigen to T cells. To migrate through the different tissues and in particular to penetrate the basement membrane, Langerhans cells are known to express collagenase IV (matrix metalloproteinase 9) (Kobayashi, 1997; Kobayashi *et al*, 1999), another enzyme associated with tumor cell migration (Liotta *et al*, 1983). Expression of Hpa1 by activated Langerhans cells could be essential to penetrate the heparan sulfate rich basement membrane between dermis and epidermis. We are investigating in our *in vitro* model (Régnier *et al*, 1997) the hapten-induced expression of Hpa1 in human epidermal Langerhans cells.

Based on our observations we suppose that Hpa1 plays an important role in epidermal physiology; however, the determination of its exact role requires further investigation. Experiments are in progress to elucidate its role in the desquamation process, including electron microscopy studies to determine its exact location within the stratum corneum. As it is known that heparan sulfate undergoes age-related changes (Feyzi *et al*, 1998), a study of the Hpa1 expression in young and aged skin is envisaged, as well as a monitoring of heparanase expression in the hair follicle, where the enzyme could be involved in the remodeling of the extracellular matrix, an important event in the hair cycle.

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